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<b>(21) International Application Number:</b> PCT/US90/03300 <b>(22) International Filing Date:</b> 11 June 1990 (11.06.90)  <b>(30) Priority data:</b> 365,291 12 June 1989 (12.06.89) US  <b>(71) Applicant:</b> RESEARCH FOUNDATION FOR MENTAL HYGIENE, INC. [US/US]; 44 Holland Avenue, Albany, NY 12229 (US).  <b>(72) Inventors:</b> ISAACS, Charles, E. ; 30 Devon Drive North, Manalapan, NJ 07726 (US). THORMAR, Halldor ; Hulduland 14, IS-100 Reykjavik (IS). KIM, Kwang, S. ; 178 Dahlia Street, Staten Island, NY 10312 (US). HEIRD, William, C. ; 39 East 75th Street, Apt 3E, New York, NY 10021 (US).		<b>(74) Agent:</b> KURUCZ, John; Kane, Dalsimer, Sullivan, Kurucz, Levy, Eisele & Richard, 711 Third Avenue, New York, NY 10017 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> REDUCING THE SPREAD OF INFECTION BY BLOOD HANDLING EQUIPMENT  <b>(57) Abstract</b> <p>This invention is directed to an antiviral and antibacterial activity of fatty acids and monoglycerides. More particularly, this invention is directed to the inactivation of enveloped viruses and the killing of cells by fatty acids and monoglycerides. The invention is also directed to an antiviral and/or antibacterial pharmaceutical compositions consisting essentially of inert pharmaceutical carriers and an effective amount of one or more compounds selected from the group consisting of fatty acids and monoglycerides thereof and to the use of said compounds in reducing the risk of infection in blood product handling procedures.</p> <p style="text-align: center; font-weight: bold; font-size: 1.2em;">BEST AVAILABLE COPY</p>		

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REDUCING THE SPREAD OF INFECTION BY BLOOD HANDLING EQUIPMENTCROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of co-pending U.S. patent application Serial No. 140,078, filed December 31, 1987.

FIELD OF THE INVENTION

This application is directed to antiviral and antibacterial activity of fatty acids and monoglycerides. More particularly, this application is directed to the inactivation of enveloped viruses and the killing of cells by fatty acids and monoglycerides, especially in blood and blood products.

BACKGROUND OF THE INVENTION

There are many published reports concerning the ability of human milk to protect the suckling infant from gastrointestinal infection. See, A.S. Cunningham, Morbidity In Breast-fed and Artificially Fed Infants, J. Pediatr., 1979, Vol. 95, p. 685-689; M.G. Myers et al., Respiratory and Gastrointestinal Illnesses In Breast- and Formula-fed Infants, Am. J. Dis. child, 1984, Vol. 138, p. 629-632; S.A. Larsen, Jr., Relation of Breast Versus Bottle Feeding To Hospitalization For Gastroenteritis In A Middle-Class U.S. Population, J. Pediatr., 1978, Vol. 92, p. 417-418; M.E. Fallot et al., Breast-feeding Reduced Incidence of Hospital Admissions For Infection In Infants, Pediatr., 1980, Vol. 65, p. 1121-1124; A.S. Cunningham, Breast-feeding and Health, J. Pediatr., 1987, Vol.

110, p. 658-659. Much of this protection has been attributed to the presence of immunoglobulins in the milk. See, G.A. Loslonsky et al., Maternal-neonatal Interactions and Human Breast Milk, In: Reproductive Immunology, N. Gleicher (ed.), New York, Alan R. Riss, 1981, p. 171-182; A.S. Goldman et al., Host defenses: Development and Maternal Contributions, In: Barness LA., ed., Advance in Pediatrics, Vol. 32, 1985, p. 71-100. However, it has also been shown that there are nonspecific factors in milk which can kill pathogens or slow their replication. Some of these protective factors are also nutrients, such as monoglycerides and fatty acids. Since human infant formula does not contain immunoglobulins, it has been assumed that it does not confer any protection against gastrointestinal infection. However, formulas do contain triglycerides which, following lipolysis in the stomach and intestine, produce free fatty acids and monoglycerides of which some have been shown to inactivate enveloped viruses and Giardia lamblia when present in human and bovine milk. See, J.K. Welsh et al., Use of Semliki Forest Virus To Identify Lipid-mediated Antiviral Activity and Anti-alphavirus Immunoglobulin A In Human Milk, Infect. Immun., 1978, Vol. 19, p. 395-401 (I); J.K. Welsh et al., Effect of Antiviral Lipids, Heat, And Freezing On The Activity of Viruses In Human Milk, J. Infect. Dis., 1979, Vol. 140, p. 322-328 (II); C.E. Isaacs et al., Membrane Disruptive Effect Of Human Milk: Inactivation of Enveloped Viruses, J. Infect. Dis., 1986, Vol. 154, p. 966-971, all of the

aforementioned articles being incorporated herein by reference.

Human milk contains a number of antiviral factors that are not immunoglobulins. See, W.A. Falkler, Jr., et al., A Lipid Inhibitor Of Dengue Virus In Human Colostrum And Milk, Arch. Virol., 1975, Vol. 47, p. 3-10; A.H. Fieldsteel, Non-specific Antiviral Substances In Human Milk Active Against Arbovirus And I Murine Leukemia Virus, Cancer Res., 1974, Vol. 34, p. 712-715; T.H. Matthews et al., Antiviral Activity In Milk Of Possible Clinical Importance, Lancet, 1976, Vol. II, p. 1387-1389; N.H. Sarkar et al., Effect Of Human Milk On The Mouse Mammary Tumor Virus, Cancer Res., 1973, Vol. 33, p. 626-629. Some of these factors are located in the nonlipid fraction of the milk, but most studies found antiviral activity associated with the lipid fraction. Antiviral lipids were best characterized by Welsh et al. (II), who found that free unsaturated fatty acids and monoglycerides in milk inactivated enveloped, but not nonenveloped, viruses.

As reported in C.E. Isaacs et al., Membrane Disruptive Effect Of Human Milk: Inactivation Of Enveloped Viruses, J. Infect. Dis., 1986, Vol. 154, p. 966-971, specifically incorporated herein by reference, the work of Welsh et al. (II) has been confirmed and extended. It was shown that lipids from fresh breast milk are not antiviral but become active against enveloped viruses upon storage at 4°C and in infant stomachs, probably by the release of fatty acids from milk triglycerides.

#### OBJECTS OF THE INVENTION

It is an object of the invention to provide fatty acids and monoglycerides having antiviral and antibacterial activity.

It is also an object of the invention to provide a method of inactivating enveloped viruses and killing cells by contacting same with fatty acids and monoglycerides.

It is a further object of the invention to provide a method of reducing infection in blood handling equipment by contacting blood to be handled with fatty acids and monoglycerides.

These and other objects of the invention will become more apparent from the discussion below.

#### BRIEF DESCRIPTION OF THE FIGURES

Figs. 1, 2, and 3 each represent a cross-sectional view of a test tube according to the invention.

Fig. 4 represents a cross-sectional view of a blood bag according to the invention.

Figs. 5a to 5c represent negative staining of VSV particles showing the effect of linoleic acid. VSV was incubated at 37°C for 30 min. in (a) MM, (b) linoleic acid (0.5 mg/ml of MM), and (c) linoleic acid (1 mg/ml of MM). Fig. 5a, normal intact particles covered with spikes; Fig. 5b, viral envelope no longer intact, allowing penetration of stain into most particles; Fig. 5c, virus particles in various stages of disintegration. Bar = 0.1  $\mu$ m.

Figs. 6a to 6d represent scanning electron micrographs of all cultures showing the effect of human milk and linoleic acid. Vero cells were

incubated at 37°C for 30 min in (a) human milk, (b) milk stored at 4°C for 4 days, (c) MM, or (d) linoleic acid (1 mg/ml of MM). Milk samples were diluted 1:5 in MM. Figs. 6a and 6c, intact cell membranes with microvilli; Figs. 6b and 6d, cell membranes partly or completely disintegrated. Bar = 1.0  $\mu$ m.

#### DISCUSSION

Many viruses that are virulent human pathogens have envelopes (pieces of membranes surrounding them). These include the AIDS virus (human immunodeficiency virus, HIV), and herpes viruses, including herpes simplex virus (HSV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), Varicella/Zoster (VZ), Marek's disease virus, equine abortion virus, and pseudorabies virus. The viral envelope is essential for infectivity. While many compounds are able to destroy the envelope and inactivate the virus, a large number have toxic side effects in the body. Fatty acids and monoglycerides are normal metabolites in the body, notably formed during the breakdown of milk products. It has been found that fatty acids and/or monoglycerides may be successfully used for antiviral and/or antibacterial activities.

Lipids in fresh milk do not inactivate viruses but become antiviral after storage of the milk for a few days at 4° or 23°C. The appearance of antiviral activity depends upon active milk lipases and correlates with the release of free fatty acids in the milk. A number of fatty acids which are normal components of milk lipids were tested against

enveloped viruses, i.e., vesicular stomatitis virus, herpes simplex virus, and visna virus, and against a nonenveloped virus, poliovirus. Short-chain and long chain saturated fatty acids had no or a very small antiviral effect at the highest concentrations tested. Medium-chain saturated and long-chain unsaturated fatty acids, on the other hand, were all highly active against the enveloped viruses, although the fatty acid concentration required for maximum viral inactivation varied by as much as 20-fold. Monoglycerides of these fatty acids were also highly antiviral, in some instances at a concentration 10 times lower than that of the free fatty acids. None of the fatty acids inactivated poliovirus. Anti-viral fatty acids were found to affect the viral envelope, causing leakage and at higher concentrations, a complete disintegration of the envelope and the viral particles. They also caused disintegration of the plasma membranes of tissue culture cells resulting in cell lysis and death. The same phenomenon occurred in cell cultures incubated with stored antiviral human milk. The antimicrobial effect of human milk lipids in vitro is therefore most likely caused by disintegration of cellular and viral membranes by fatty acids.

#### 1. MATERIALS AND METHODS

Cell Cultures. Vero cells (African green monkey kidney cell line; Flow Laboratories Inc., McLean, Va.) were grown in Eagle basal medium (BME) (GIBCO Laboratories, Grand Island, N.Y.) with 10% inactivated fetal bovine serum (GIBCO). Sheep



fibroblast cultures were obtained from the choroid plexus of a lamb brain and grown in 15% lamb serum (Colorado Serum Co.) in BME. The maintenance medium (MM) for Vero cells was BME with 2% fetal bovine serum; for sheep cells, the MM was 2% lamb serum in BME. Gentamicin (0.1%) was added to all media.

Viruses. Vesicular Stomatitis Virus (VSV) strain Indiana and Herpes Simplex Virus Type 1 (HSV-1) strain MacIntyre were obtained from the American Type Culture Collection, Rockville, Md., and grown in Vero cells. Visna virus strain K796 was grown in sheep choroid plexus cells. Poliovirus type 1 strain Chat was obtained from R.I. Carp (New York Institute of Basic Research) and grown in Vero cells.

Virus Titration. Viruses were titrated by inoculation of 10-fold dilutions (VSV, poliovirus, and HSV-1 were inoculated into Vero cell cultures, and visna virus was inoculated into sheep choroid plexus cell cultures) in 96-well microtiter tissue culture plates (Becton Dickinson Labware, Oxnard, Calif.). A virus dilution (0.1 ml) in MM was inoculated into each well with four wells per dilution. The plates were kept for 2 to 12 days, depending on the virus, and examined daily for cytopathic effect. Virus titers were calculated by the method of Reed and Muench (L. J. Reed et al., Am. J. Hyg., 1938, Vol. 27, p. 493-497).

Milk Samples. Human milk samples 1, 2, and 3 were collected under sterile conditions 1 to 5 months

postpartum and kept deepfrozen at  $-86^{\circ}\text{C}$  until used in experiments.

Reagents. Fatty acids and monoglycerides were purchased from Sigma Chemical Co., St. Louis, Mo. (purest grade). Monoglyceride ethers were custom synthesized by Deva Biotech, Inc., Paoli, Penna. Immediately before use they were melted and emulsified in liquid form in BME with 10% fetal bovine serum by vortexing at the highest speed for 1 min. The emulsions (100 mg/ml) were diluted to the desired concentrations in MM. Emulsions of shortchain fatty acids were neutralized to pH 7 by addition of 1 M NaOH. Unsaturated fatty acids and monoglycerides were kept under nitrogen, and emulsions were used within a few minutes of preparation. Eserine sulfate (physostigmine; Sigma) and NaCl were dissolved in water and diluted in MM before use in experiments.

Assay of Antiviral Activity. About  $10^5$  50% tissue culture infective doses ( $\text{TCID}_{50}$ ) of virus were mixed with a five-fold dilution of milk in MM or with an emulsion of fatty acids and monoglycerides in MM and incubated at  $37^{\circ}\text{C}$  for 30 min. Virus mixed with MM alone was used as a control. After incubation, the infectivity of each mixture was titrated by the serial dilution endpoint method. Dilutions (10-fold) were made in MM. The  $10^2$  to  $10^5$  dilutions were inoculated into monolayers of Vero cells, and the virus titers were determined as described above. The difference between the titer ( $\log_{10}$ ) of the control virus and the titers of milk-virus and

lipid-virus mixtures, i.e., the reduction of virus titer, was used as a measure of antiviral activity.

Preparation of Virus For Electron Microscopy.

VSV was concentrated and partially purified by differential centrifugation in a Beckman L2-65B ultracentrifuge, and samples ( $10^{10}$  TCID<sub>50</sub>/ml) were incubated at 37°C for 30 min. in MM with or without emulsified fatty acids. The virus suspensions were applied to carbon-coated grids and negatively stained with 2% phosphotungstic acid, pH 7.0. Specimens were examined by using a Hitachi HS 8-2 electron microscope at 50 kV.

Preparation of Cells For Electron Microscopy.

Monolayer cultures of cells were incubated for 30 min. at 37°C either in MM alone or with milk or a fatty acid emulsion. The cell layers were then carefully rinsed with Hanks balanced salt solution and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer. After rinsing in buffer and postfixation with 2% osmium tetroxide, the cells were dehydrated through gradings of ethanol, critical-point dried, and sputter coated with 10.5 nm of gold. They were examined in an ISI-ISS40 scanning electron microscope at 20 kV.

Estimation Of Free Fatty Acids Levels. Lipids from 100  $\mu$ l of the milk samples were extracted with 0.5 ml of chloroform-methanol (2:1). The upper phase was removed, and an aliquot of the chloroform layer was separated by thin-layer chromatography on Silica Gel G (Merck & Co., Inc., Rahway, N.J.) plates with quantitative standards of oleic acid in a solvent system consisting of hexane-diethylether-acetic acid

(70:30:1.5). The developed plates were charred after spraying with dichromatesulfuric acid, and the free fatty acids were quantitated by densitometry.

## 2. RESULTS

### Relationship Between Lipolysis and Antiviral

Activity. Previous results (Isaacs et al.) showed that human milk becomes active against enveloped viruses after storage at 4°, 23°, or -20°C for various lengths of time. The antiviral activity is associated with the cream fraction, but the skim fraction is needed for the lipids to become antiviral. To test whether the appearance of antiviral activity depended on active milk lipases, milk samples 1, 2, and 3 were stored at 4°C for 4 days with or without two lipase inhibitors, 5 MM eserine sulfate and 1 M NaCl. The virus titer (VSV) fell from  $10^5$  to  $\leq 10^{1.5}$  TCID<sub>50</sub> after incubation with milk stored without an inhibitor, thus showing a reduction of  $10^{3.5}$  TCID<sub>50</sub>. In contrast, virus incubated in the same way with milk which had been stored with lipase inhibitors showed no loss of infectivity at the concentrations used. The inhibitors had no effect on milk which was already antiviral.

Another indication that the appearance of antiviral activity in stored human milk is associated with lipolysis is shown in the following table:

TABLE 1. Free Fatty Acids (FFA) and Antiviral Activity in Milk<sup>a</sup>

Milk Sample	Storage		Reduction of		Lipoprotein lipase (U/ml)
	temp/time (°C/days)	FFA (mg/ml)	VSV titer (log <sub>10</sub> )	_____	
1	-86/4	0.5	0		336
	23/4	12.0	4.0		
	4/4	7.0	4.0		
3	-86/4	0.5	0		20
	23/4	2.0	0		
	4/4	2.0	0		

a The same results were obtained for milk tested fresh or after storage at -86°C.

According to Table 1, deep-frozen human milk sample 1 did not have a detectable level of free fatty acids, but the level increased to 7 and 12 mg/ml upon storage at 4° and 23°C, respectively, for 4 days. Both stored samples were highly anti-viral. The free fatty acid, level of milk sample 3, on the other hand, increased to only 2 mg/ml upon storage, and the milk did not become antiviral. Compared with milk sample 3, milk sample 1 had much higher levels of lipoprotein lipase, which was previously shown to correlate with the appearance of milk antiviral activity.

Antiviral Activity of Fatty Acids and Monoglycerides. A comparison of the antiviral activity of a number of fatty acids found in milk is shown in the following table:

TABLE 2. Viral Inactivation By Incubation With Fatty Acids At 37° For 30 min.

Fatty Acid	Concn <sup>a</sup> in mg/ml (MM)	Reduction of virus titer (log10)		
		VSV	HSV-1	VV <sup>b</sup>
Butyric (4:0) <sup>c</sup>	10 (113)	0	ND <sup>d</sup>	ND
Caproic (6:0)	10 (86)	0	ND	ND
Caprylic (8:0)	10 (69)	1.8	ND	≥3.2
Capric (10:0)	4 (22)	≥4.0 <sup>e</sup>	≥4.0	≥3.2
Lauric (12:0)	2 (10)	≥4.0	≥4.0	≥3.2
Myristic (14:0)	4 (16)	≥4.0	≥4.0	1.7
Palmitic (16:0)	20 (78)	1.0	1.0	0.7
Palmitoleic (16:1)	2 (15)	≥4.0	≥4.0	≥3.2
Stearic (18:0)	20 (70)	0	ND	ND
Oleic (18:1 cis)	2 (7)	≥4.0	≥4.0	≥3.2

TABLE 2. Viral inactivation by incubation with fatty acids at 37° for 30 min.

Fatty Acid	Concn <sup>a</sup> in mg/ml (MM)	Reduction of virus titer (log <sub>10</sub> )		
		VSV	HSV-1	VV <sup>b</sup>
Butyric (4:0) <sup>c</sup>	10 (113)	0	ND <sup>d</sup>	ND
Caproic (6:0)	10 (86)	0	ND	ND
Caprylic (8:0)	10 (69)	1.8	ND	≥3.2
Capric (10:0)	4 (22)	≥4.0 <sup>e</sup>	≥4.0	≥3.2
Lauric (12:0)	2 (10)	≥4.0	≥4.0	≥3.2
Myristic (14:0)	4 (16)	≥4.0	≥4.0	1.7
Palmitic (16:0)	20 (78)	1.0	1.0	0.7
Palmitoleic (16:1)	2 (15)	≥4.0	≥4.0	≥3.2
Stearic (18:0)	20 (70)	0	ND	ND
Oleic (18:1 cis)	2 (7)	≥4.0	≥4.0	≥3.2



CON'T.

TABLE 2. Viral inactivation by incubation with fatty acids at 37° for 30 min.

<u>Fatty Acid</u>	<u>Concn<sup>a</sup> in mg/ml (MM)</u>	<u>Reduction of virus titer (log<sub>10</sub>)</u>		
		<u>VSV</u>	<u>HSV-1</u>	<u>VV<sup>b</sup></u>
Elaidic (18:1 trans)	2 (7)	≥4.0	ND	ND
Linoleic (18:2)	1 (3.5)	≥4.0	≥4.0	≥3.2
Linolenic (18:3)	1 (3.6)	≥4.0	≥4.0	≥3.2
Arachidonic (20:4)	0.5 (1.6)	≥4.0	ND	ND

a Concentration of fatty acid in virus mixtures incubated at 31°C for 30 min. All fatty acids were tested in a series of twofold concentrations. Shown is either the lowest concentration which reduced the VSV titer by  $\geq 4.0 \log_{10}$  units of the highest concentration tested (butyric, caproic, caprylic, palmitic, and stearic).

b VV, Visna virus.

c Carbon atoms:double bonds.

d ND, Not done.

e The titer ( $\log_{10}$ ) of the control virus incubated with mM was 5.5, whereas no virus was detectable in the  $10^{-2}$  to  $10^{-5}$  dilutions of fatty acid-virus mixtures. It was not possible to test these mixtures in lower dilutions ( $10^{-1}$  or undiluted) because they were toxic to the cell cultures. Assuming that the  $10^{-1}$  dilution contained infectious virus, the highest possible titer of the fatty acid-virus mixture was  $10^{1.5}$  TCID<sub>50</sub>, and the reduction of virus titer ( $\log_{10}$ ) would equal 4.0 (5.5 minus 1.5). If the titers of the mixtures were less than  $10^{1.5}$ , the reduction of titer would be greater than 4.0. It can be seen from Table 2 that short-chain (butyric, caproic, and caprylic) and long-chain saturated (palmitic and stearic) fatty acids had no or a very small antiviral effect at the highest concentrations tested. On the other hand, the medium-chain saturated and long-chain unsaturated fatty acids were all antiviral but at different concentrations. Table 2 shows the lowest concentration causing a 10,000-fold reduction in VSV titer. A 2-fold-lower concentration either did not

inactivate the virus or caused only a 10-fold reduction in titer. Similar results were obtained for HSV-1 and visna virus, a retrovirus. In contrast, incubation of poliovirus at 37°C for 30 min. with capric, lauric, myristic, palmitoleic, oleic, linoleic, linolenic, and arachidonic acids, each at a concentration of 8 mg/ml, did not cause a significant reduction of virus titer compared with the titer of poliovirus incubated without fatty acids ( $10^{4.7}$  TCID<sub>50</sub>). The sodium salts of oleic and linoleic acids had antiviral effects similar to those of the free acids.

Other products of lipolysis, e.g., 1-monoglycerides of fatty acids, were also tested for antiviral activity, as shown in the following table:

Table 3. Viral Inactivation In Human Serum By Incubation With  
Monoglycerides At 37°C For 30 Min.

<u>Monoglyceride</u>	<u>Concn<sup>a</sup> in mg/ml (MM)</u>	<u>Reduction of Virus titer (log<sub>10</sub>)</u>	
		<u>VSV</u>	<u>HSV-1</u>
Monocaprylin (8:0) <sup>b</sup>	2.0 (9)	≥4.0	ND <sup>c</sup>
Monocaprin (10:0)	0.5 (2)	≥4.0	≥3.7
Monolaurin (12:0)	0.25 (0.9)	≥4.0	≥3.7
Monomyristin (14:0)	2.0 (13)	3.0	ND
Monolein (18:1)	1-0 (2.8 <sup>d</sup> )	2.3	ND
Monolinolein (18:2)	0.25 (0.7)	≥4.0	ND

- a Lowest concentration causing  $\geq 3.0 \log_{10}$  in reduction in virus titer.
- b Carbon atoms:double bonds.
- c ND, Not done.
- d Highest antiviral activity of the concentrations tested (0.5 to 4 mg/ml). The same results were obtained when the monoglyceride was dissolved in ethanol and diluted 1:100 in mm before being added to the virus.

All the monoglycerides tested except monomyristin and monoolein were antiviral in concentrations 5 to 10 times lower (millimolar) than those of the corresponding fatty acids.

The above experiments with human milk, milk stomach contents, and purified lipids show that MGs and fatty acids which are released from human milk triglycerides either during storage or in the gastrointestinal tract kill enveloped viruses and very likely serve an in vivo protective role in breast-fed infants.

Studies have also been done to determine the time required for viral inactivation. Virus was incubated with monolaurin (12:0) in maintenance media:

TABLE 3ATime Course of Viral Inactivation

<u>Incubation Time (min)</u>	<u>Reduction of HSV-1 titer</u>
30	≥4.0
10	≥4.0
5	≥4.0
1	≥4.0
0.5	≥4.0

These results indicate that viral killing is rapid and probably happens as the MG or FFA comes into contact with the viral envelope. Electron micrographs with negative staining of VSV incubated with linoleic acid showed that at 0.5 mg per ml leakage of viral envelopes was produced allowing the stain to enter many particles. The effect was far more pronounced with 1 mg of linoleic acid per ml, causing particle disintegration.

Effect Of Fatty Acids On Viral Particles. To study the effect of fatty acids on virus particles, VSV was concentrated, partly purified, and then incubated at 37°C for 30 min. in MM with or without linoleic acid. Negative staining of virus incubated without fatty acids showed an abundance of characteristic bullet shaped particles covered with spikes and containing coiled nucleocapsids (Fig. 5a). Incubation with 0.5 mg of linoleic acid per ml caused leakage of viral envelopes, allowing the stain to enter many particles (Fig. 5b). The effect

was far more pronounced with 1 mg of linoleic acid per ml (Fig. 5c), causing particle disintegration. Titration of the samples used for electron microscopy showed a 10-fold reduction in virus titer with 0.5 mg of linoleic acid per ml, whereas 1 mg/ml caused a  $\geq 1,000$ -fold reduction. Similar results were obtained by negative staining of VSV incubated with low concentrations of arachidonic acid.

Disintegration Of Cell Membranes By Fatty Acid.

Negative staining of VSV treated with fatty acids suggested that virus inactivation results from disruption of the viral envelope, which is derived from the host cell plasma membrane. To study the effect on cell membranes, monolayers of Vero cells or sheep fibroblasts were incubated at 37°C for 30 min. in MM with or without 1 mg of linoleic acid per ml and examined by scanning electron microscopy. Control cells incubated in MM without fatty acids showed intact cell membranes (Fig. 6c), whereas in cell layers treated with 1 mg of linoleic acid per ml, the cell membranes were partly or completely disintegrated (Fig. 6d), causing cell lysis. The same effect was seen by incubation of cells with human milk which had been stored at 4°C for 4 days (Fig. 6b). This milk sample (no. 1) (Table 1) contained 7 mg of fatty acids per ml and was highly antiviral. On the other hand, milk sample 1 stored at -86°C for 4 days (Table 1) showed no effect on cell membranes (Fig. 6a).

The micrographs in Figs. 5a to 5c reflect negative staining of VSV particles showing the effect of linoleic acid treatment. Titration of the

samples used for electron microscopy showed a  $\leq 10$ -fold reduction in virus titer with 0.5 mg of linoleic acid per ml whereas 1 mg/ml caused a  $\geq 10,000$ -fold reduction. Similar results were obtained by negative staining of VSV incubated with low concentrations of arachidonic acid.

It was next examined whether the effects of antiviral fatty acids were additive so that changes in the concentration of one antiviral component in a mixture can be compensated for by increasing or adding another fatty acid. Mixtures of fatty acids were made in which individual fatty acid concentrations had been found to either not inactivate the virus, or to reduce the titer by less than 10-fold. Mixtures were incubated with virus in maintenance medium. The results are set forth in the following table:



Table 4. Antiviral Activity of Fatty Acid Mixtures

<u>Fatty Acid Mixture</u>	<u>Individual Fatty Acid Conc. (mg/ml)</u>	<u>Total Fatty Acid Conc. (mg/ml)</u>	<u>Reduction of VSV titer (<math>\log_{10}</math>)</u>
Capric	2	3	$\geq 3.7$
Lauric	1		
<u>Lauric</u>	1	2	$\geq 3.7$
Myristic	1		
<u>Lauric</u>	1	2	$\geq 3.7$
Oleic	1		
<u>Oleic</u>	1	1.5	$\geq 3.7$
Linoleic	0.5		
<u>Lauric</u>	0.7		
Oleic	0.7	1.7	$\geq 3.7$
Linoleic	0.3		

The ability to make antiviral mixtures of medium and long-chain fatty acids indicates that a balance can be made between the potentially toxic effects of high concentrations of medium chain fatty acids in vivo and the loss of antiviral long-chain fatty acids by binding to serum albumin and other blood proteins.

Effect of Antiviral Milk Samples on HIV Titers.

Human milk and stomach contents samples that have been found to kill HSV-1 and VSV were tested against HIV (AIDS virus). The results are set forth in the following table:

Table 5. HIV Inactivation by Antiviral Human Milk

<u>Sample</u>	<u>Storage</u>	<u>Reduction of HIV titer (<math>\log_{10}</math>)</u>
1	Fresh	0
1A	4°C	5.0
2	Fresh	0
2A	4°C	5.0
3	Fresh	0
3A	4°C	3.5
4	Fresh	0
4A	Stomach Contents (3 hrs)	3.0

As with assays of other enveloped viruses, HIV was diluted five-fold with sIgA depleted milk or stomach contents. Therefore, anti-HIV activity in the undiluted sample is greater than the 1,000 to

100,000-fold reduction in titer in the assay mix. The results also show that HIV is as sensitive to inactivation by milk lipids as the other enveloped viruses that were tested. It should, therefore, be possible to screen large numbers of lipid mixtures against HSV-1, for example, which is much less expensive to assay than HIV and then test only the promising mixtures against HIV.

Effect Of An Antiviral Monoglyceride On CMV Titers.

Monocaprin (10:0), which had previously been found to inactivate HSV-1 at a concentration of 2 MM, was tested against three separate CMV strains.

Incubations were performed in a maintenance medium containing 10% serum. The results are set forth in the following table:

Table 6. Inactivation of CMV by a Purified Lipid

<u>CMV Strain Tested</u>	<u>Reduction of CMV Titer (<math>\log_{10}</math> TCID 50%)*</u>
AD 169	$\geq 3.69$
Espilat	$\geq 3.50$
Towne	$\geq 2.67$

\* TCID 50% - Tissue culture infective dose 50%, expressed as  $\log_{10}$ .

The above results establish that CMV as well as HSV-1, HIV, and other enveloped viruses can be inactivated in a serum-containing medium.

Monoglyceride Inactivation of HSV-1 in Human Serum.

HSV-1 was added directly to human serum, and virus inactivation was measured in the presence of either monocaprin (10:0) or monolaurin (12:0). The results are set forth in the following table:

Table 7. HSV-1 Inactivation In Human Serum

<u>Monoglyceride Added*</u>	<u>Conc. (mg/ml)</u>	<u>Reduction in HSV-1 titer (log<sub>10</sub>)</u>
Control	-	0
Monocaprin	1	0.8
Monocaprin	2	1.8
Monocaprin	4	≥4.0
Monolaurin	1	0.8
Monolaurin	2	1.5
Monolaurin	4	2.0

\* The incubation mixture contained human serum, HSV-1 (titer 5.5), and the indicated monoglyceride.

Monolaurin at 4 mg/ml reduced serum HSV-1 titer by only 100-fold whereas monocaprin at the same concentration decreased the viral titer by ≥10,000-fold. In our in vitro studies, monolaurin had more antiviral activity on a concentration basis (millimolar) than monocaprin. The serum results suggest that nonspecific interactions in serum and presumably plasma and other blood products are as important as inherent antiviral activity for determining which monoglycerides to add to human

blood and blood products to inactivate viral pathogens.

Monoglyceride Inactivation of HSV-1 In Infant Formula. When monoglycerides were added to another complex fluid, infant formula (Enfamil), differences in HSV-1 killing were found as they were in human serum. The results are set forth in the following table:

Table 8. HSV-1 Killing in Infant Formula

<u>Monoglyceride Added*</u>	<u>Conc. (mg/ml)</u>	<u>Reduction in HSV-1 titer (log<sub>10</sub>)</u>
Monocaprin	0.5	0
Monocaprin	1	0.3
Monocaprin	2	2.3
Monolaurin	0.5	0.3
Monolaurin	1	0.3
Monolaurin	2	1.0
Monoolein	0.5	0
Monoolein	1	0
Monoolein	2	0
Monolinolein	0.5	0
Monolinolein	1	0.3
Monolinolein	2	0.5

\* The incubation mixture contained formula, HSV-1, (titer 5.5), and the indicated monoglyceride.

As can be seen from the results set forth in Table 8, in infant formula, as in human serum, monocaprin appears to be the most effective monoglyceride against enveloped viruses. In maintenance medium monolinolein produced the same

reduction in viral titer as monacaprins but at one-third the concentration (millimolar). Monocaprins at the concentration used was over 60-fold more effective in infant formula than monolinolein.

Effect Of Added Monoglyceride On RBCs In Human Blood. A monocaprins concentration of 3 mg/ml that had previously been shown to be antiviral was added to whole human blood samples, and red blood cell counts were compared to those in the same sample before lipid addition. The results are set forth in the following table:

Table 9. Stability of Red Blood Cells to Added Monoglyceride

<u>Sample</u>	<u>Red Blood Cells*</u>	
	<u>Untreated</u>	<u>Treated</u>
1	4.59	4.46
2	5.10	4.78
3	5.30	5.19
4	4.94	4.74
5	5.08	4.36

\* Units -  $10^3/\text{mm}^3$

The results show that a monocaprins concentration that will kill  $\geq 4.0 \log_{10}$  of enveloped virus when added to human serum does not lyse RBCs in whole blood.

Antibacterial Effect of Human Milk and Purified Monoglycerides. Fatty acids and monoglycerides are antibacterial as well as antiviral. Stomach contents (supplied by Dr. William C. Heird, Columbia Presbyterian Medical Center) from infant fed human milk by gastric intubation were tested for

antibacterial activity against *Staphylococcus epidermidis* (gram +), *Escherichia coli* (gram -) and *Salmonella enteritidis* (gram -). See the following table:

Table 10. Antiviral and Antibacterial Activity  
of Human Milk Stomach Contents\*

Log<sub>10</sub> Reduction in Bacterial\*\*  
Titer by 1 Hour Stomach Contents

Sample	Log <sub>10</sub> Reduction in HSV-1 Titer by 1 Hour Human Stomach Contents		S. epidermidis E. coli		S. enteritidis	
1	24.0	-	25.0	-	24.0	-
2	24.0	25.0	24.0	-	-	-
3	24.0	24.0	-	-	-	-
4	24.0	-	-	-	-	-

\* The milks themselves were all tested for antiviral and antibacterial activity, and none was detected.

\*\* Each sample was not tested against all the bacterial strains because there was not a sufficient volume of stomach contents.



Stomach contents that were antiviral were also antibacterial, killing both gram+ and gram- bacteria. Since human milk contains both medium chain and long-chain fatty acids, it was next determined whether gram+ and gram- bacteria were equally sensitive to different chain lengths. The results are set forth in the following table:

Table 11. Inactivation of Gram+ and Gram- Bacteria by Monoglycerides

Reduction of bacterial concentration (log <sub>10</sub> )						
	E. <sup>2</sup> <u>coli</u>	S. <sup>2</sup> <u>enteritidis</u>	H. <sup>2,3</sup> <u>influenza</u>	S. <sup>4</sup> <u>epider- midis</u>	GroupB <sup>4</sup> <u>strepto- coccus</u>	
Monocapryloyl (8:0)	≥5.0	-	≥8.0	≥4.0	-	32
Monocaprin (10:0)	≥5.0	-	≥8.0	≥4.0	4.5	
Monolaurin (12:0)	0	0	≥8.0	≥4.0	4.5	
Monolein (18:1)	0	0	-	≥4.0	-	
Monolinolein (18:2)	-	-	-	-	4.5	
Monelcosenoin (20:1)	0	0	-	≥4.0	-	

1 Each MG was used at 2 mg/ml.

2 Gram-  
3 Hemophilus influenza  
4 Gram+

Gram positive bacteria were inactivated comparably by medium chain saturated and long-chain unsaturated monoglycerides. However, the gram - bacteria *E. coli* and *S. enteritidis* were unaffected by long-chain unsaturated fatty acids and monolaurin. *H. influenza* was inactivated by monolaurin so that there are differential sensitivities to MGs between different gram - bacteria. Differences in bacterial inactivation may be due to the bacterial wall, membrane or both. Scanning electron micrographs (not shown) of *S. epidermidis* treated with monolaurin showed that the bacteria were completely disintegrated. It is therefore possible to manipulate MGs and their concentrations to lyse some membranes and leave others intact.

Human milk becomes antiviral not only upon storage but also in the stomach of infants within one hour of feeding. The appearance of antiviral activity in stored milk is related to the level of lipoprotein lipase in the milk, indicating that it is caused by the release of fatty acids or other products of lipid hydrolysis. Similar results were previously reported by Welsh et al. (I, II). Data herein indicate that the antiviral effect of stored human milk is caused by lipolysis, and of the nine fatty acids most commonly found in human milk, seven are highly active in killing enveloped viruses. The polyunsaturated long-chain fatty acids were the most active, but medium-chain saturated fatty acids, particularly lauric and myristic acids, also showed

activity. Monocaprin and monolaurin were active in concentrations ten times lower than those of the corresponding free acids, but monomyristin was consistently less active. Long-chain saturated fatty acids, which make up about 30% of the fatty acids in human milk, and short-chain fatty acids, which are more common in cow milk, were not, or were very slightly, antiviral. The concentrations of fatty acids found to reduce viral titers by  $\geq 10,000$ -fold in vitro (Table 2) are in the same range of fatty acid concentrations found in human milk. The results indicate that as lipolysis of milk triglycerides proceeds, either during storage or in the gastrointestinal tract, two types of antiviral lipids, monoglycerides and free fatty acids, are produced. It is possible that these two classes of lipid differ in efficacy against intestinal pathogens. This may also be true for the members of each lipid class.

The results are similar to those of earlier studies with different viruses and further establish the marked antiviral effect of most fatty acids found in milk. The electron microscope study suggests that the antiviral effect is caused primarily by disintegration of viral envelopes by fatty acids. Similar findings were reported by Sarkar et al., who treated mouse mammary tumor virus with the cream fraction of human milk and noted degradation of the viral envelope. Our study also shows disintegration of the plasma membrane of cultured cells with concentrations of fatty acids and stored human milk that inactivate enveloped

viruses. The fatty acids and monoglycerides which have been found to be strongly antiviral were shown to induce fusion of cell membranes. Although the exact mechanism is not clear, it has been suggested that the fatty acids and their monoesters are incorporated into the lipid membrane, causing destabilization of the bilayer. A similar mechanism might lead to the complete disintegration of cell membranes and viral envelopes we observed. We did not compare the sensitivity of cultured cells and enveloped viruses at various fatty acid concentrations.

Several studies have indicated a lower incidence of infections, particularly gastrointestinal, in breast-fed versus bottle-fed infants. However, the role of milk fatty acids and their derivatives in protecting babies against illness is not established, despite their well-known antimicrobial effect in vitro. Although most known gastrointestinal viruses are nonenveloped, necrotizing enterocolitis in infants is caused by an enveloped virus, i.e., a human enteric coronavirus. *Giardia lamblia*, an intestinal protozoan parasite infecting children, is killed by milk fatty acids in vitro, suggesting the possibility of a giardiacidal effect of fatty acids in the intestines. Since fatty acids lyse cells by disrupting their plasma membranes, it is likely that they kill not only *giardia* but also other parasitic protozoa. Although a few studies have demonstrated antimicrobial activity of human and animal stomach contents after milk feeding, much more work is needed to

characterize the active factors and to establish their role in prevention of, and recovery from, gastrointestinal infections.

It is within the scope of the invention that fatty acids and/or monoglycerides thereof are used for antiviral and/or antibacterial activity. The compounds used can be selected from the group consisting of saturated or unsaturated fatty acids having from 4 to 22 carbon atoms and esters of glycerol with said acids. Preferred compounds comprise saturated fatty acids having from 4 to 14 carbon atoms, particularly from 6 to 14 carbon atoms, and monoglycerides thereof. Especially preferred are  $C_7 - C_{12}$  fatty acid monoglycerides, either singly or in mixtures thereof. Also useful according to the invention are mono- or polyunsaturated fatty acids having from 14 or 16 to 22 carbon atoms, especially from 14 to 18, 16 to 18 or 16 to 20 carbon atoms, and the monoglycerides thereof. The above-mentioned ranges of carbon atoms are inclusive of fatty acids having odd numbered carbon atoms.

It is also within the scope of the invention to employ ether and/or lysophosphatidylcholine derivatives of fatty acids having antiviral and/or antibacterial activity. For example, useful fatty acid derivatives would have an ether bond between a fatty acid and glycerol. Examples of such compounds include 1-O-decyl-SN-glycerol, 1-O-lauryl-SN-glycerol, 1-O-octyl-SN-glycerol, and 1-O-oleyl-SN-glycerol. Useful lysophosphatidylcholine derivatives include, for

example, L- $\alpha$ -lysophosphatidylcholine caproyl, L- $\alpha$ -lysophosphatidylcholine decanoyl, and L- $\alpha$ -lysophosphatidylcholine lauroyl. Also, the fatty acids useful according to the invention can be used in the form of their pharmacologically acceptable salts, such as alkali metal salts. Useful examples of such salts include the sodium and lithium salts.

The compounds according to the invention can be used singly or in mixtures. For example, it is preferred that from 1 to 6 compounds, especially from 1 to 4 compounds, be administered at one time.

The results of testing reflecting the usefulness of monoglyceride ethers and lysophosphatidylcholine derivatives are shown in the following tables:

Table 12. Inactivation of Vesicular Stomatitis Virus By  
Monoglyceride Ethers In Human Plasma<sup>1</sup>

<u>Monoglyceride Ether</u>	<u>Concentration</u> <u>(MM)</u>	<u>Sodium</u> <u>Tauchocholate</u>	<u>Reduction in VSV</u> <u>Titer (Log<sub>10</sub>)</u> —
1-O-Decyl-SN-glycero <sup>3</sup>	5	+	0
1-O-Octyl-SN-glycerol <sup>2</sup>	10	-	≥4.0
	10	-	2.0
	15	-	≥4.0
1-O-Octyl-SN-glycerol	5	+	≥4.0
1-O-Octyl-SN-glycerol	10	-	1.3
	15	-	≥4.0
1-O-Oleyl-SN-glycerol <sup>4</sup>	5	-	0
	10	-	0
	15	-	0

1 Incubations were done at 37° for 30 minutes.

2 8 carbon ether.

3 10 carbon ether.

4 18 carbon ether.



Table 13. The Antiviral Activity of Monoglyceride Esters and Ethers Is Additive<sup>1</sup>

Ether (Carbons)	Concn. (MM)	Ester (Carbons)	Concn. (MM)	Sodium Taurocholate	Concn. (MM)	Reduction in VSV Titer (log <sub>10</sub> )
8	5	8	5	-	-	0
8	7.5	8	7.5	-	-	3.7
8	5	8	5	+	5	≥4.0
8	2.5	8	2.5	+	10	≥4.0
10	5	10	5	-	-	2.0
10	7.5	10	7.5	-	-	1.8
10	5	10	5	+	5	≥4.0
10	2.5	10	2.5	+	10	≥4.0
8	2.5	8	2.5)	-	-	1.3
10	2.5	10	2.5)	-	-	-
8	2.5	8	2.5)	+	5	≥4.0
10	2.5	10	2.5)	+	5	≥4.0

<sup>1</sup> Incubated at 37° for 30 minutes in human plasma.

Table 14. Time Course Of VSV Inactivation  
At 37° In Human Plasma With 15  
MM 1-O-octyl-SN-glycerol

<u>Incubation Time (min.)</u>	<u>Reduction in VSV Titer (Log<sub>10</sub>)</u>
2.5	0
5	≥4.0
10	≥4.0
15	≥4.0
30	≥4.0

The 8 carbon and 10 carbon MG ethers are just as effective as the naturally occurring esters, and, in fact, the 8 carbon derivative appears to be somewhat more antiviral than the 8 carbon ester. The 18 carbon ether (Table 12) showed the same lack of antiviral activity as the ester in human plasma.

Table 15. Antiviral Activity of Lysophosphatidylcholine Derivatives

<u>Lipid</u>	<u>Conc. (MM)</u>	<u>Sodium Taurocholate (10MM)</u>	<u>Log<sub>10</sub> Reduction in VSV Titer</u>
L- $\alpha$ -Lysophosphatidylcholine caproyl (8C)	5	+	0
	5	-	0
	10	+	0
	10	-	0
	15	+	1.0
L- $\alpha$ -Lysophosphatidylcholine decanoyl (10C)	15	-	0
	5	+	.7
	5	-	1.7
	10	+	1.7
	10	-	1.0
L- $\alpha$ -Lysophosphatidylcholine lauroyl (12C)	5	+	1.7
	5	-	1.2
	10	+	2.0
	10	-	2.0

The activity of the aforesaid fatty acids and/or monoglycerides (esters) can be enhanced by the presence of blood enzyme lipase (LPL) inhibitors. LPL breaks down the preferred fatty acid monoglycerides into constituent free fatty acids and glycerol, which is significant because while the free fatty acids are also antiviral, they require much higher concentration than the corresponding monoglycerides to be effective in human blood. LPL inhibitors reduce or deter such breakdown. Useful LPL inhibitors include, for example, the following:

- 1) Bile salts or acids and conjugated bile salts or acids, at concentrations of from about 1 nanomolar to 1 molar. An example of a conjugated bile acid is taurocholic acid, the sodium and lithium salts of which are readily available. Bile salts are also detergents, and therefore they provide additional antiviral activity;
- 2) Sodium chloride, at concentrations of from about 1 micromolar to 10 molar; and
- 3) Protamine chloride or sulfate, at concentrations of from about 1 nanomolar to 1 molar.

When LPL inhibitors are used with, for example, monoglycerides according to the invention, they can be used in an MM:MM ratio of from about 1:1 to 1:6, based upon the monoglyceride or monoglycerides used. Preferably this ratio is from about 1:1.5 to 1:4.

To demonstrate the effectiveness of, for example, the conjugated bile acid taurochloric acid (sodium salt), an incubation mixture containing herpes antibody negative human serum, herpes simplex virus-1 (titer) 5.5, and a monoglyceride, was

prepared. A quantity of 12 MM (final concentration) sodium taurochlorate was added to some of the samples. The results were as follows:

Table 16

Herpes Monoglyceride <u>Added</u>	Sodium <u>Taurocholate</u>	Reduction in Simplex Virus-1 <u>Titer (<math>\log_{10}</math>)</u>
1-Monocapryloyl-rac- Glycerol (8:0) 7.5MM	+ -	$\geq 4.0$ 0
1-Monodecanoyl-rac- Glycerol (10:0) 7.5MM	+ -	$\geq 4.0$ 0
1-Monodecanoyl-rac- Glycerol (10:0) 15MM	+ -	$\geq 4.0$ 3.0

In analogous procedures additional testing of the effectiveness of sodium taurocholate in human serum was performed. The results are set forth in the following tables:

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Table 17

1-Monocapryloyl- rac-glycerol (MM)	1-Monodecanoyl- rac-glycerol (MM)	Sodium Taurocholate (MM)	Reduction in VSV Titer (log <sub>10</sub> )
7.5	-	12	≥5.0
-	7.5	12	≥5.0
7.5	-	10	≥5.0
-	7.5	10	≥5.0
7.5	-	8	≥5.0
-	7.5	8	≥5.0
7.5	-	6	≥5.0
-	7.5	6	≥5.0
7.5	-	4	4.0
-	7.5	4	≥5.0
7.5	-	2	≥3.0
-	7.5	2	≥5.0
7.5	-	0	≥1.0
-	7.5	0	1.0

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Table 18

1-Monolauroyl-rac-glycerol (MM)	Sodium Taurocholate (MM)	Reduction in VSV Titer ( $\log_{10}$ )
15	10	$\geq 5.0$
15	-	0
7.5	10	$\geq 5.0$
7.5	-	0
5	10	$\geq 5.0$
5	-	0
4	10	$\geq 5.0$
4	-	0
3	10	$\geq 5.0$
3	-	0

Table 17 shows that when 1-Monocapryloyl-rac-glycerol and 1-Monodecanoyl-rac-glycerol monoglycerides are used with varying concentrations of sodium taurocholate, the 1-Monodecanoyl-rac-glycerol is effective when used with concentrations as low as 2 MM sodium taurocholate whereas the 1-Monocapryloyl-rac-glycerol needs at least 6 MM sodium taurocholate to be effective. The 1-Monocapryloyl-rac-glycerol monoglyceride is still more likely to be used in a product, however, because it is more soluble.

Table 18 shows that the 1-Monolauroyl-rac-glycerol monoglyceride also kills vesicular stomatitis virus at concentrations as low as 3 MM in the presence of 10 mm sodium taurocholate. Also, Table 18 shows that viral killing by 1-Monolauroyl-rac-glycerol monoglyceride only takes place when sodium taurocholate is present.

Further testing was conducted to demonstrate the effectiveness of sodium taurocholate in human plasma. The results were as follows:



Table 19

1-Monocapryloyl-rac-glycerol (MM)	1-Monodecanoyl-rac-glycerol (MM)	Sodium Tauracholate (1 MM)	Reduction in VSV titer (log <sub>10</sub> )
4	-	10	2.0
4	-	-	0
7.5	-	10	≥4.0
7.5	-	-	1.0
15	-	10	≥4.0
15	-	-	2.0
-	4	10	1.0
-	4	-	0
-	7.5	10	≥4.0
-	7.5	-	1.0
-	15	10	≥4.0
-	15	-	≥4.0

The additional antiviral activity in the presence of sodium or lithium taurocholate is due not only to LPL inhibition but also to detergent activity of these compounds. In fact, the results presented below show that in phosphate buffer as little as 5MM sodium taurocholate can inactivate VSV. However, in human plasma a sodium taurocholate concentration of at least 20MM is required to kill  $\geq 4.0 \log_{10}$  VSV, and therefore taurocholate by itself may not be desirable for use in human blood and blood products used for transfusion.

Table 20. Inactivation Of VSV By Sodium Taurocholate  
In Phosphate Buffer and Human Plasma

Sodium Taurocholate (MM)	Reduction of VSV titer ( $\log_{10}$ )	
	Phosphate Buffer	Plasma
0	0	0
2.5	1.0	0
5	$\geq 4.0$	0
10	$\geq 4.0$	0
15	$\geq 4.0$	1.0
20	$\geq 4.0$	3.7
		49

The importance of LPL inhibitors for the antiviral activity of monoglycerides depends upon the endogenous LPL activity in each blood sample. Since LPL activity is variable and each blood sample will not be tested for LPL activity, it would be preferred to add LPL inhibitors and monoglycerides to each clinical blood sample to ensure maximum antiviral activity. Also, since the addition of lithium taurocholate does not interfere with any clinical assays tested, it provides added antiviral activity with no drawbacks.

Fatty acids and monoglycerides thereof are readily available. Should it be necessary, desired monoglycerides can be prepared from the corresponding fatty acid or acids by esterification with glycerol according to known procedures.

The above-described compounds have demonstrated antiviral and/or antibacterial activity. It is within the scope of this invention that virus or bacteria-containing media, such as blood, can be treated with an effective amount of one or more fatty acids and/or monoglycerides thereof. It is also within said scope that a human or warm-blooded animal having a viral or bacterial condition may be treated for said condition by administration of a composition according to the invention.

For treatment or prophylaxis purposes one or more of the compounds described herein can be administered to a human or warm-blooded animal perorally, parenterally, intravenously, topically, or rectally as active ingredient in customary pharmaceutical compositions, that is, compositions

consisting essentially of an inert pharmaceutical carrier and an effective amount of active ingredient, such as tablets, coated pills, capsules, wafers, powders, solutions, suspensions, emulsions, suppositories, creams, and the like. An effective amount for such application of the compounds according to the present invention would be on the order of from about 0.001 to 4 mg/kg of body weight, preferably from about 0.01 to 3 mg/kg of body weight, 1 to 4 times daily.

The naturally occurring ester derivatives are antiviral and do not interfere with the clinical assays examined with the exception of triglyceride measurements. Since triglyceride concentration is determined by the enzymatic release of fatty acids from the glycerol backbone, monoglycerides push these measurements off scale on the high side. By using ether linkages rather than ester linkages, the fatty acid remains attached to the glycerol backbone because lipases do not work on ether linkages and the triglyceride measurements are unaffected.

While, as shown above, sodium taurocholate is effective as an LPL inhibitor and has antiviral activity, other physiologically acceptable salts, such as lithium salts, are notable. By use of lithium taurocholate instead of the sodium salt, any interference with blood sodium measurements has been eliminated. In some testing a combination of monoglyceride ether and lithium taurocholate was in excess of what is needed to inactivate  $\geq 4 \log_{10}$  of any enveloped virus in all of the blood and blood products examined.

Further testing has shown the effect of monoglyceride esters and ether on the total white blood cell concentration in whole human blood. The results of said testing are as follows:

Table 21. Stability of White Blood Cells In Whole Human Blood To  
Added Lipid

<u>Sample</u>	<u>Concentration (MM)</u>	<u>Total White Blood Cells</u>
Control	--	6.4
1-Monocapryloyl-rac-glycerol	15	2.4
1-O-Octyl-SN-glycerol	15	0.6
1-Mmonodecanoyl-rac-glycerol	15	0.7
1-O-Decyl-SN-glycerol	15	1.31

To further appreciate the invention, it should be noted that monoglycerides added to human blood and human serum have been found to inactivate viruses present yet have no adverse effect on the serum or the red blood cells. This is of particular interest in blood handling procedures and equipment.

8 to 10 carbon monoglyceride (MG) at a concentration of 4 mg/ml did not interfere with 17 commercially available Abbott's diagnostic test kits and MA bioproduct test kits. The Abbott's kits tested were: HBsAg (hepatitis B surface antigen); Anti-HBc (anti-hepatitis B core antigen); Anti-HBs (anti-hepatitis B surface antigen); Anti-HBcIgM (anti-hepatitis B core IgM); Anti-HAVIgM (hepatitis Be antigen); Anti-HBe (anti-hepatitis Be antigen); Anti-delta hepatitis. The MA Bioproduct ELISA kits tested were: mumps antibody; Herpes simplex type I antibody; Herpes Simplex type II antibody; Toxoplasmosis antibody; CMV antibody; Rubella antibody; Measles antibody; and Chlamydia antibody. Positive and negative test results remained consistent among the control and mg treated samples. The test results were as follows:



(1) HBsAg (Hepatitis B Surface Antigen) Test

	<u>HBsAg</u>	
	<u>Untreated<sup>1</sup></u>	<u>ETOH<sup>2</sup> Treated (ETOH with MG)<sup>3</sup></u>
Patient #1 (positive)	129	145
Patient #2 (positive)	39.4	30
Patient #3 (negative)	0.89	0.60
Cont. A (-) <sup>4</sup>	79	
Cont. B (+) <sup>4</sup>	5,600	0.82

1 Untreated: use serum or controls without adding anything.

2 ETOH: add 1  $\mu$ l of ETOH to 100  $\mu$ l of serum and then vortex mix this mixture before running the test.

3 Treated: add 1  $\mu$ l of ETOH containing MG to 100  $\mu$ l of serum and then vortex mix this mixture before running the test.

4 Both positive and negative control samples do not need treatment. Therefore, use the same controls used in regular runs.

(2) Anti-HBC (Anti-hepatitis B Core Antigen)Anti-HB Core

	<u>Untreated</u>	<u>ETOH</u>	<u>Treated (ETOH with MG)</u>
Patient #4 (positive)	33	28	22
Patient #5 (positive)	42	41	33
Patient #6 (negative)	0.73	0.60	0.54
Cont. A (-)	1.9		
Cont. B (+)	0.06		

(3) Anti-HBsAg (Anti-Hepatitis B Surface Antigen)HBsAg

	<u>Untreated</u>	<u>ETOH</u>	<u>Treated (ETOH with MG)</u>
Patient #7 (positive)	227	247	313
Patient #8 (positive)	48.3	51	61
Cont. A (-)	79		
Cont. B (+)	5,926		

(4) Anti-HBcIgM (Anti-hepatitis B core IgM)Anti-HB Core IgMUntreated ETOH Treated (ETOH with MG)

Patient #9 (positive)	1.045	1.014	1.176
Patient #10 (positive)	1.024	1.243	1.044
Patient #11 (negative)	0.146	0.124	0.038
Cont. A (-)	0.581		
Cont. B (+)	1.058		

(5) Anti-HAV IgM (Anti-hepatitis A IgM)HAVAB IgMUntreated ETOH Treated (ETOH with MG)

Patient #12 (positive)	0.548	0.593	0.586
Patient #13 (positive)	0.359	0.322	0.334
Patient #14 (negative)	0.047	0.054	0.051
Cont. A (-) #0.037	0.083		
Cont. B(+) #1.025	1.187		

(6) Anti-HAV (Anti-hepatitis) A VirusHAVABUntreated ETOH Treated (ETOH with MG)

Patient #15 (positive)	0.028	0.039	0.027
Patient #16 (positive)	0.020	0.019	0.023
Patient #17 (negative)	1.303	1.173	1.110
Cont. A (-)	1.170		
Cont. B (+)	0.18		

(7) HBeAg (Hepatitis Be Antigen)HBeAgUntreated ETOH Treated (ETOH with MG)

Patient #18 (positive)	0.849	0.853	0.752
Patient #19 (positive)	0.778	0.945	0.955
Patient #20 (negative)	0.020	0.015	0.095
Cont. A (-)	0.078		
Cont. B (+)	0.987		

(8) Anti HBe (Anti-hepatitis Be antigen)Anti-HBeUntreated ETOH Treated (ETOH with MG)

Patient #21 (positive)	0.060	0.053	0.058
Patient #22 (positive)	0.064	0.070	0.061
Patient #23 (negative)	0.364	0.524	0.417
Cont. A (-)	0.619		
Cont. B (+)	0.088		

(9) Anti-delta hepatitisAnti-DeltaUntreated ETOH Treated (ETOH with MG)

Patient #24 (positive)	0.018	0.022	0.029
Patient #25 (positive)	0.030	0.030	0.033
Patient #26 (negative)	0.716	0.768	0.942
Cont. A (-)	1.166		
Cont. B (+) #	0.028		

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(10) MA Bioproduct ELISA Test for Mumps Antibody

	<u>Untreated</u>	<u>ETOH</u>	<u>Treated (ETOH with MG)</u>
Patient #7 (positive)	227	247	313
Patient #8 (positive)	48.3	51	61
Cont. A (-)	79		
Cont. B (+)	5,926		

(11) MA Bioproduct ELISA Test for Herpes Simplex Type

	<u>Untreated</u>	<u>ETOH</u>	<u>Treated (ETOH with MG)</u>
Patient #30 (+)	1.22	0.970	1.15
Patient #31 (+)	0.920	0.820	0.810
Patient #32 (+)	0.060	0.060	0.070
Cont. A (+)	0.530		
Cont. B (-)	0.070		

(12) MA Bioproducts ELISA Test for Herpes Simplex Type II

	<u>Untreated</u>	<u>ETOH</u>	<u>Treated (ETOH with MG)</u>
Patient #33 (+)	1.600	1.600	1.550
Patient #34 (+)	0.350	0.320	0.270
Patient #35 (+)	0.040	0.045	0.050
Cont. A (+)	0.530		
Cont. B (-)	0.040		

(13) MA Bioproduct ELISA Test For Toxoplasmosis Antibody

	<u>Untreated</u>	<u>ETOH</u>	<u>Treated (ETOH with MG)</u>
Patient #36 (+)	1.300	1.110	1.240
Patient #37 (+)	1.310	1.290	1.350
Patient #38 (+)	0.070	0.000	0.000
Cont. A (+)	0.580		
Cont. B (-)	0.000		

(14) MA Bioproduct ELISA Test For CMV Antibody

	<u>Untreated</u>	<u>ETOH</u>	<u>Treated (ETOH with MG)</u>
Patient #39	1.874	1.802	1.785
Patient #40 (+)	0.900	0.971	0.889
Patient #41 (+)	0.023	0.005	0.010
Cont. A (+)	0.580		
Cont. B (-)	0.000		

(15) MA Bioproduct ELISA Test For Rubella Antibody

	<u>Untreated</u>	<u>ETOH</u>	<u>Treated (ETOH with MG)</u>
Patient #42 (+)	0.95	0.49	0.63
Patient #43 (+)	0.50	0.44	0.50
Patient #44 (+)	0.12	0.12	0.08
Cont. A (+)	0.60		
Cont. B (-)	0.00		



(16) MA Bioproduct ELISA Test For Measles Antibody

	<u>Untreated</u>	<u>ETOH</u>	<u>Treated (ETOH with MG)</u>
Patient #45 (+)	0.22	0.17	0.20
Patient #46 (+)	0.27	0.25	0.25
Patient #47 (+)	0.065	0.065	0.065
Cont. A (+)	0.43		
Cont. B (-)	0.00		

(17) MA Bioproduct ELISA Chlamydia Antibody Test

	<u>Untreated</u>	<u>ETOH</u>	<u>Treated (ETOH with MG)</u>
Patient #48 (+)	0.325	0.270	0.280
Patient #49 (+)	0.385	0.460	0.440
Patient #50 (+)	0.090	0.090	0.080
Cont. A (+)	0.450		
Cont. B (-)	0.000		

Addition of fatty acids did not interfere with routine blood tests. However, the addition of monoglyceride esters interferes with triglyceride measurements. The use of monoglyceride ethers eliminates this problem.

Also, additional testing has shown that the presence of LPL inhibitors, such as are described above, does not interfere with the results and/or effectiveness of the above-described test kits.

Certain aspects of the invention can be more readily appreciated by reference to Figs. 1 to 4. In Figs. 1 to 3, a test tube 1, a representative example of blood handling equipment, may comprise at its closed end 2 an effective amount of liquid or powder active ingredient 3. After a quantity of a blood product such as blood serum (not shown) is introduced into test tube 1, the open end 4 is closed with a stopper 5. Preferably the test tube and stopper are then shaken for a sufficient amount of time to cause active ingredient 3 to dissolve in the blood serum. In the alternative, the blood serum could be stirred or agitated with an appropriate agitating means (not shown) to cause active ingredient 3 to dissolve. It is a preferred embodiment of the invention that the active ingredient instantaneously or substantially instantaneously deactivates undesired viruses or bacteria.

The liquid or powder 3 may comprise active ingredient alone or active ingredient in combination with suitable physiologically or pharmacologically acceptable carrier or diluent. It is within the

scope of the invention that the liquid or powder 3 could be, with or without carrier or diluent, in the form of a capsule or pill (not shown) formed from conventional ingredients. The pill could be coated or uncoated; however, if the pill has a coating, the coating would preferably be a readily soluble or dissolvable coating, in the same manner that the exterior capsule material would necessarily be readily soluble or dissolvable.

The liquid or powder 3, whether in pill or capsule form or not, can be added either prior to or after introduction of a blood product. For example, a premeasured quantity of powder or a pill could be added to a test tube containing a blood product.

In Fig. 2 the interior surface 10 of the test tube 1 is coated with a coating 11 comprising an effective amount of active ingredient and suitable carrier. The interior surface 10 can be either partly or wholly coated, depending upon the particular carrier and active ingredient used. A partial coating 11 could be continuous or discontinuous. It is preferred that at least the interior surface of the closed end 2 be coated since this surface is most likely to be in contact with blood serum introduced. The coated test tube and blood can be agitated or shaken as described above.

If a test tube or other blood handling container is to have a coating, the coating would comprise active ingredient as well as a carrier comprised of any of the materials known in the art for this purpose. For example, the coating could comprise a readily soluble polymeric substance useful for this

purpose, such as a cellulose-based polymeric material or a hydrophilic acrylate or methacrylate polymer or copolymer. See, for example, U.S. Patents Nos. 3,566,874, 3,695,921, 4,156,066, 4,156,067, and 4,417,892, each of which is incorporated herein by reference.

The test tube system shown in Fig. 3 comprises a coated cartridge or substrate 15. The coating on the cartridge 15 contains active ingredient and a suitable carrier. When a blood product such as blood serum (not shown) is introduced into test tube 16, the coating dissolves in said blood. Test tube 16 has a stopper 17 so that the blood serum and cartridge can be shaken. Optionally test tube 16 could also have a coating (not shown) as described above.

Cartridge or substrate 15 may be comprised of any suitable material. For example, substrate 15 may be dissolvable or nondissolvable, sterilizable or inherently sterilized, hydrophilic, or coated or impregnated. It is important that said material not interfere, either physically or analytically, with further processing of the blood product. The actual materials, as well as the amounts to be used, impregnated, or coated, can be readily determined.

Fig. 4 comprises a blood bag 20 which can have active ingredient either as powder (not shown) or a coating 21 on the interior surface 22 of the blood bag 20. Said coating can, be continuous or discontinuous, whole or partial. Dissolution of the coating and active ingredient can be enhanced by

shaking or other agitation of the blood bag with blood inside.

It is within the scope of the invention that other methods and arrangements or configurations can be used to distribute active substance in a blood product handling piece of equipment or hardware, particularly one having flexible sides. See, for example, U.S. Patents Nos. 2,800,905, 3,312,221, 3,818,910, 4,236,517, and 4,417,892, each of which is incorporated herein by reference, which disclose other arrangements or configurations to have an active ingredient available to be dissolved in liquid.

When compounds according to the invention are applied for treatment or prophylaxis reasons to test tubes or other conventional laboratory, hospital, or medical containers or hardware for the treatment and/or storage of blood, plasma, serum, or other blood product, the compounds should be applied in doses more concentrated than doses administered to a patient. The ratio of treated material (MG) to blood sample in the Abbot and MA Bioproduct commercial test kit trials was 1:100. The envisioned ratio of the MG compounds administered to other medical containers or hardware is from about 10  $\mu$ g to 500 mg/ml of blood, plasma, serum, or other blood product, preferably from about 25  $\mu$ g/ml to 200 mg/ml of blood, plasma, serum, or other blood product, more preferably from about 75  $\mu$ g/ml to 100 mg/ml of blood, plasma, serum, or other blood product. Examples of other conventional containers or hardware include blood bags, vacutainer tubes,

test kit materials, veno claysis kits, rubber gloves, and the like. An exemplary aspect of the invention would be precoated vacutainer tubes.

The aforesaid invention need not be limited to treatment of blood products or body sera. Other potential applications or the antiviral and antimicrobial monoglycerides and fatty acids include: facial cream (as an acne treatment), bactericidal, fungicidal, virucidal; shampoo, hand lotion; athlete's foot medication (ointment, powder, soap); candies (for sore throat, bad breath, recurrent herpes); ointment or foam spray (for genital herpes lesion treatment); shaving cream; mouth wash; after shave lotions; tooth paste; diaper rash preventer; plasma bag treatment; disposable glove treatment; additive to pasteurized cow milk; additive to blood sample tubes to inactivate HIV and CMV (safety measure for lab technicians); additives for condoms, band-aids, or bandages; additive for paint; or animal treatment for viral infections.

A particularly useful application of the invention herein is the treatment of rubber gloves. Rubber gloves could be "dusted" on inner and/or outer surfaces with powdered antiviral component according to the invention. In a further embodiment, the gloves themselves could be comprised of a material, preferably polymeric, containing antiviral component that would release antiviral component upon contact with liquid such as a blood product or other body fluid or derivative thereof.

The preceding specific embodiments are illustrative of the practice of the invention. It

is to be understood, however, that other expedients known to those skilled in the art or disclosed herein may be employed without departing from the spirit of the invention or the scope of the appended claims.

W E C L A I M:

1. An improved blood product holding means, wherein the improvement comprises the presence of an antiviral component comprising an antiviral effective amount of one or more compounds selected from the group consisting of  $C_4 - C_{14}$  fatty acids and monoglycerides thereof and  $C_{14} - C_{22}$  mono- or polyunsaturated fatty acids and monoglycerides thereof.

2. The holding means of Claim 1 which comprises a test tube a blood bag, or a vacutainer set.

3. The holding means of Claim 1, wherein the holding means has an internal coating containing the antiviral component.

4. The holding means of Claim 1, wherein the antiviral component is in powder or liquid form.

5. The holding means of Claim 1, wherein the antiviral component is contained in or on a substrate.

6. The holding means of Claim 1 which also comprises an effective amount of LPL inhibitor.

7. The holding means of Claim 6, wherein the LPL inhibitor is sodium or lithium taurocholate.

8. The holding means of Claim 1, wherein the antiviral component comprises one or more compounds selected from the group consisting of  $C_7 - C_{12}$  fatty acids and monoglycerides thereof.

9. The holding means of Claim 8, wherein the antiviral component comprises monocaprylin or monocaprin.



10. The holding means of Claim 1, wherein the antiviral component comprises from 1 to 4 compounds.

11. The holding means of Claim 1, wherein the fatty acids of the antiviral component are saturated fatty acids.

12. The holding means of Claim 1, wherein the fatty acids of the antiviral component are mono- or ~~polyunsaturated fatty acids.~~

13. The holding means of Claim 1 which comprises rubber gloves comprising antiviral component.

14. The holding means of claim 13, wherein the rubber gloves have antiviral component-containing powder thereon.

15. The holding means of Claim 1, wherein the blood product is blood, plasma, or blood serum.

16. A method of treating a sample of blood product to be tested or handled to arrest antiviral activity of said sample, which comprises contacting said sample with an antiviral component which comprises an antiviral effective amount of one or more compounds selected from the group consisting of  $C_4 - C_{14}$  fatty acids and monoglycerides thereof and  $C_{14} - C_{22}$  mono- or polyunsaturated fatty acids and monoglycerides thereof.

17. The method of Claim 16, wherein the antiviral component also comprises an effective amount of LPL inhibitor.

18. The method of Claim 17, wherein the LPL inhibitor is sodium or lithium taurocholate.

19. The method of Claim 16, wherein the antiviral component comprises one or more compounds

selected from the group consisting of  $C_7 - C_{12}$  fatty acids and monoglycerides thereof.

20. The method of Claim 19, wherein the antiviral component comprises monocaprylin or monocaprin.

21. The method of Claim 16, wherein the antiviral component comprises from 1 to 4 compounds.

22. The method of Claim 16, wherein the fatty acids of the antiviral component are saturated fatty acids.

23. The method of Claim 16, wherein the fatty acids of the antiviral component are mono- or polyunsaturated fatty acids.

24. The method of Claim 16, wherein said sample is contacted with said antiviral component in blood handling means.

25. The method of Claim 24, wherein said blood handling means is a test tube, blood bag, or vacutainer set.

26. In a test kit for testing a blood product sample, the improvement which comprises an antiviral component comprising an effective antiviral amount of one or more compounds selected from the group consisting of  $C_4 - C_{14}$  fatty acids and monoglycerides thereof and  $C_{14} - C_{22}$  mono- or polyunsaturated fatty acids and monoglycerides thereof.

27. In a combination for handling blood product, samples of blood product, or samples of another body fluid or derivative thereof, said combination comprising one or more sample containers and processing apparatus, the improvement which

comprises at least one of said sample containers comprising an antiviral component comprising an anti-viral effective amount of one or more compounds selected from the group consisting of  $C_4 - C_{14}$  fatty acids and monoglycerides thereof and  $C_{14} - C_{22}$  mono- or polyunsaturated fatty acids and monoglycerides thereof.

28. In a material used in a medical or hospital setting, the improvement which comprises at least one surface of said material comprising an antiviral component comprising an antiviral effective amount of one or more compounds selected from the group consisting of  $C_4 - C_{14}$  fatty acids and monoglycerides thereof and  $C_{14} - C_{22}$  mono- or polyunsaturated fatty acids and monoglycerides thereof.

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FIG. 1

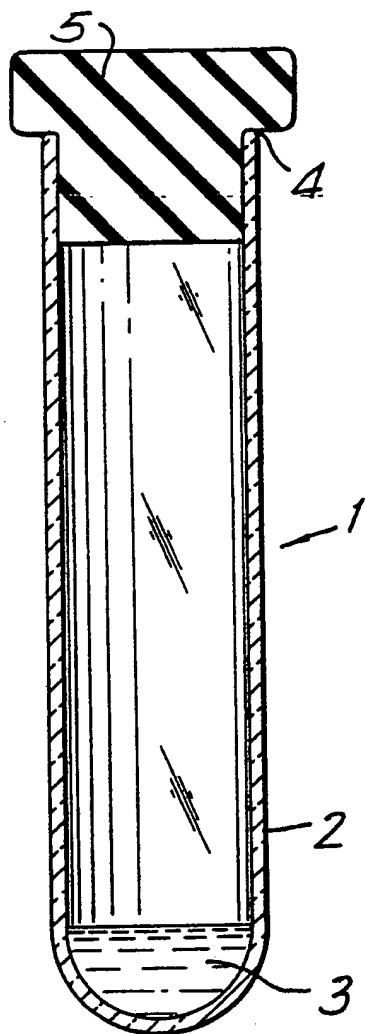
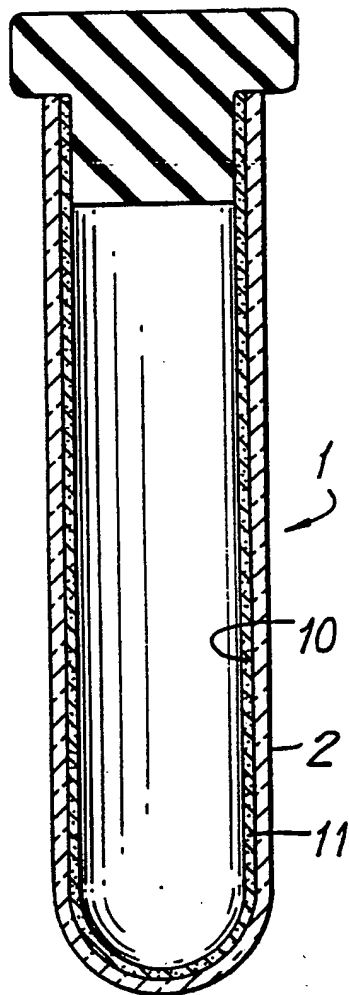


FIG. 2



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FIG. 3

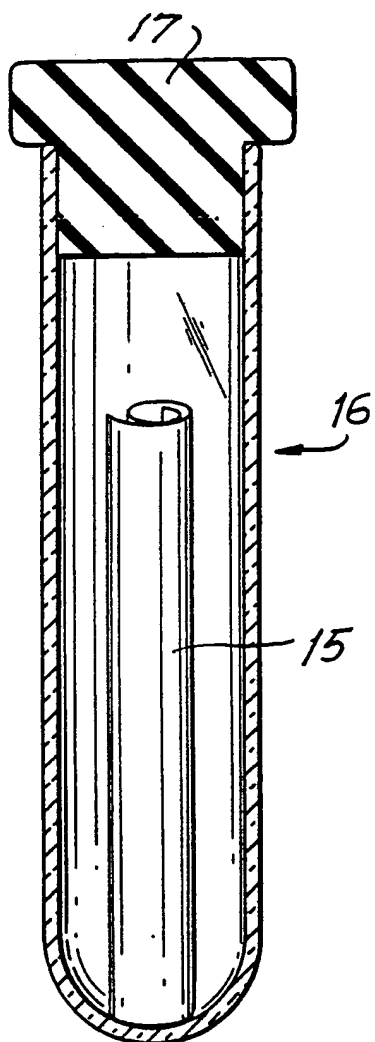
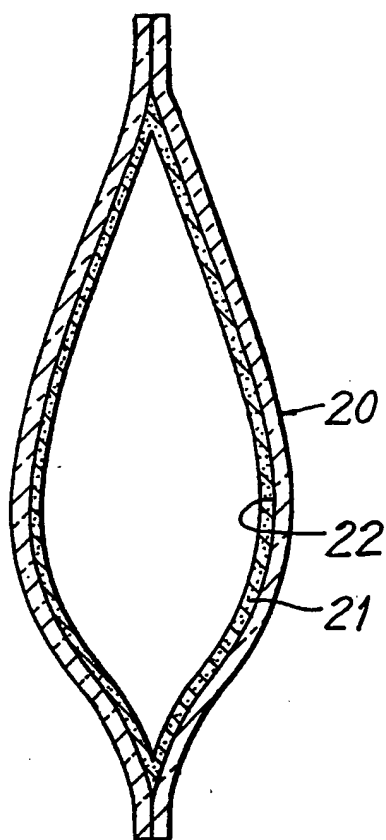
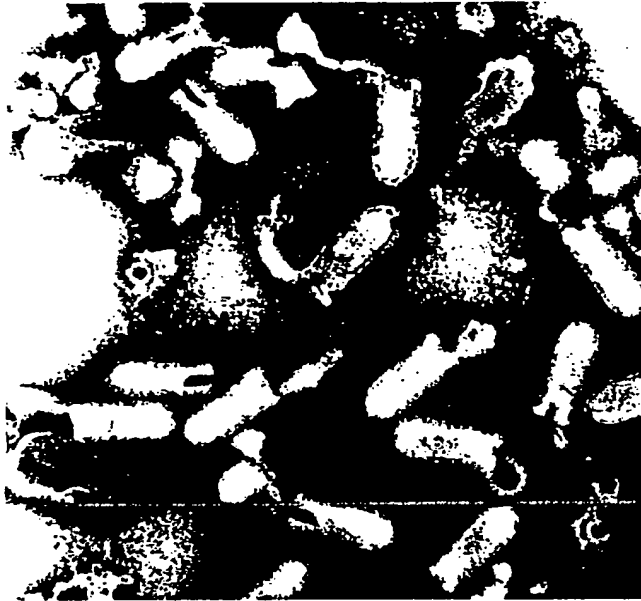


FIG. 4



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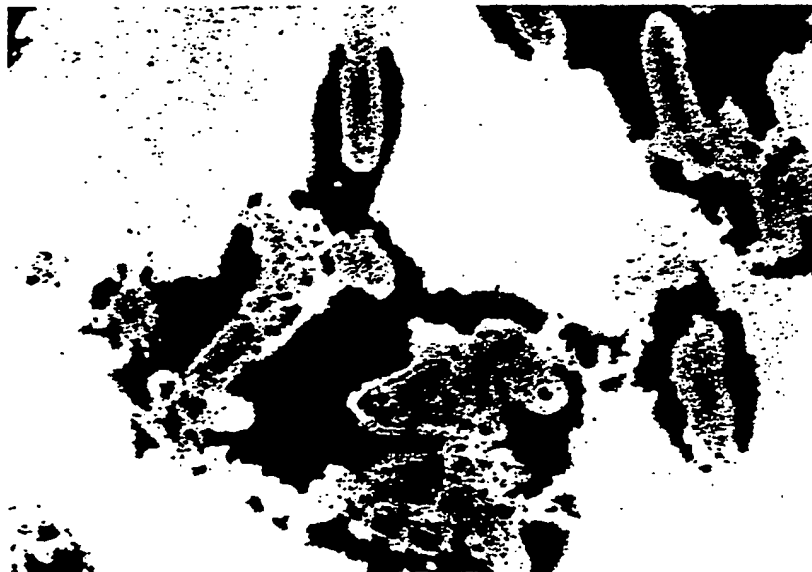
*FIG.5a*



*FIG.5b*



*FIG.5c*



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FIG. 6b

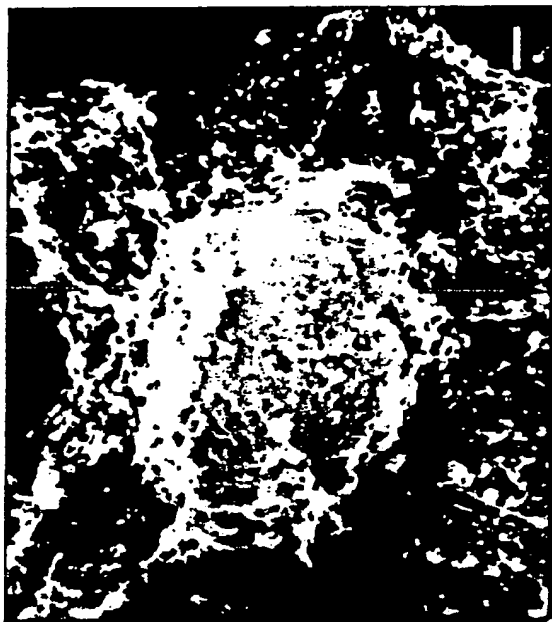


FIG. 6d

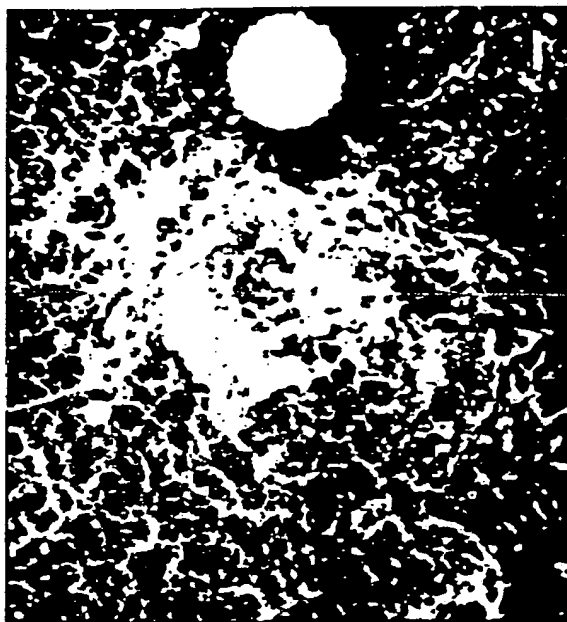


FIG. 6a

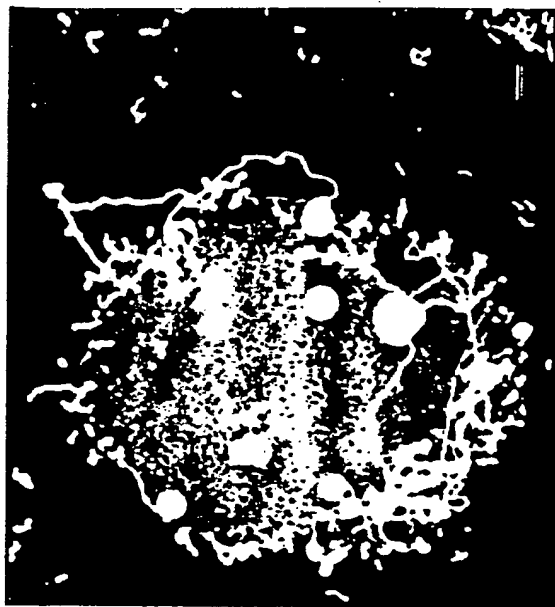
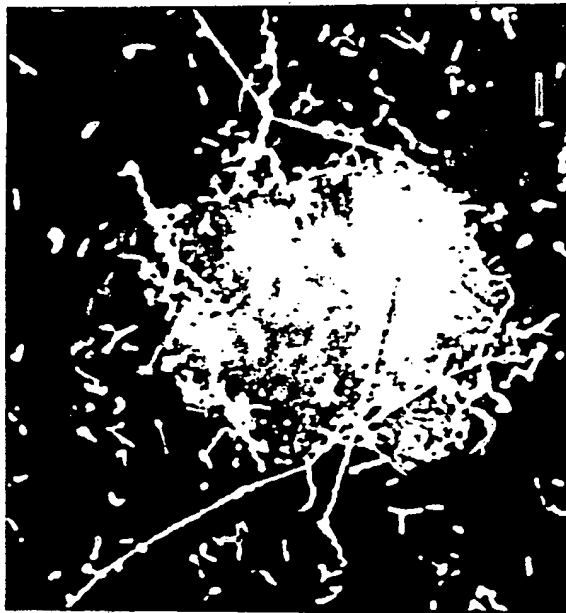


FIG. 6c



# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US90/03300**

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5) : A61K 31/22, 31/225, 31/19, 31/20 U.S. Cl : 514/546, 547, 549, 552, 557, 558							
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; margin-top: 10px;">Minimum Documentation Searched <sup>4</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 50%; text-align: left; padding: 5px;">Classification System <sup>1</sup></th> <th style="width: 50%; text-align: left; padding: 5px;">Classification Symbols</th> </tr> <tr> <td style="padding: 5px;">U.S.</td> <td style="padding: 5px;">514/546, 547, 549, 552, 557, 558</td> </tr> </table> <div style="text-align: center; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>5</sup></div>				Classification System <sup>1</sup>	Classification Symbols	U.S.	514/546, 547, 549, 552, 557, 558
Classification System <sup>1</sup>	Classification Symbols						
U.S.	514/546, 547, 549, 552, 557, 558						
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>							
Category <sup>6</sup>	Citation of Document, <sup>14</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>		Relevant to Claim No. <sup>14</sup>				
Y	US,A 4,513,008 (REVICI) 23 April 1985 See column 2, lines 63-68 and column 5, lines 55-68.		1-28				
Y	US,A 4,806,352 (CANTRELL) 21 February 1989 See column 3, lines 13-29 and column 5, lines 3-17.		1-28				
<div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p><sup>15</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>							
<b>IV. CERTIFICATION</b>							
Date of the Actual Completion of the International Search <sup>2</sup>		Date of Mailing of this International Search Report <sup>3</sup>					
20 SEPTEMBER 1990		6 NOV 1990					
International Searching Authority <sup>1</sup>		Signature of Authorized Officer <sup>18</sup>					
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